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GLYCOGEN IN THE NERVOUS SYSTEM.

I. METHODS FOR LIGHT AND ELECTRON MICROSCOPY

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It is recognized that glycogen is normally present in the nervous tissue.[32] It is also known that it appears in different areas of the nervous system in many pathological conditions.[1,6,13,18,23,27,33] Recently, it has been observed that the increase of glial glycogen is one of the earliest signs of radiation injury.[16,17,21,24,25] As a prerequisite for the study of these changes at the ultrastructural level, it seems pertinent to determine the best methods of tissue preparation. Despite the abundant literature, there is no complete agreement regarding the different problems of adequate fixation and identification of glycogen. Alcoholic fixatives were considered the most adequate for the histochemical preservation of glycogen. Among them, the picroalcoholic fixatives, such as the Gendre, Duboscq-Brazil, and Rossman, have been extensively used in glycogen histochemistry. The Rossman fluid insures the highest amount of glycogen preservation in the fixed tissue according to Graumann,[14] Grillo,[15] and Swigart.[34] However, alcoholic or picroalcoholic fixatives are not suitable for ultrastructural studies. On the other hand, the pattern of distribution and the coarse granular appearance

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of glycogen after fixation by those fluids is far apart from that expected in the living cell.

An ideal method of fixation must preserve the maximum amount of glycogen in the tissue and keep a morphologically correct image of the actual distribution of the glycogen in tissue elements as well as its relationships to different cell organelles. The fixative should allow for the preparation of enzymatic control sections and the tissue must be able to "take" appropriate stains. To fulfill these purposes, different methods of fixation have been used (Table I). The differences between these methods is really one of grade rather than one of nature. For instance with cryofixation one cannot avoid the use of chemicals. On the other hand, the best chemical fixations are achieved at low temperature.

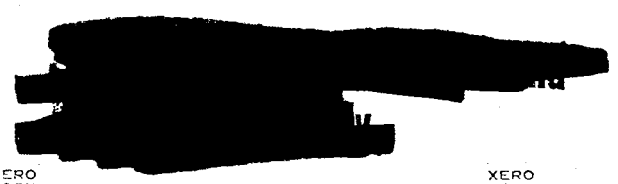
Table I

In the present report we will refer only to chemical procedures. Even in this restricted field there is disagreement. Nevertheless there are some routines that enjoy the preference of most light and electron microscopists (Table II). If these routines are compared with each other, they are so far apart that at first it is difficult to understand that they are meant to achieve similar goals. It is widely assumed that for light microscopy, the alcoholic fixatives are superior to aqueous fixatives for glycogen. On the other hand, all the ultrastructural studies done so far have been performed with aqueous fixatives, such as solutions of permanganate and osmium tetroxide.

Table II

MATERIAL AND METHODS

Brain and spinal cord from 25 newborn White Leghorn chickens and 20 adult Sprague-Dawley rats were used to study normal tissue and induced



pathological conditions (wounds, burns, and radiation injury). Tissues were processed for light and electron microscopy. Some of the rats were killed by decapitation, immediately the calvaria was opened and small slices of brain fixed by immersion in cold Rossman fluid and kept in the ice box for 24 or 48 hours, followed by the routine procedures for paraffin embedding for light microscopy studies. Following similar procedure, the glycogen bodies of some chickens were also fixed by immersion in cold Rossman. In some cases intravascular perfusion with the same fixative was performed for preservation of rat brain and chicken spinal cord. As an aqueous fixative, chromic osmium solution[7] was utilized with a slight modification of Palay's technique.[7,28] Furthermore, tiny pieces of rat brain and chicken spinal cord were fixed by immersion in the same fixative.

The animals were anesthetized with sodium nembutal. An intravenous inoculation of 0.5 ml of a 1-percent solution of sodium nitrite was done in the femoral vein, the inoculation needle being previously rinsed with heparin. After 15 or 20 minutes, the anterior part of the chest was opened, the pericardium was incised and a small canule introduced through the left ventricle to the first portion of the aorta. The perfusion starts with a small amount of warm Ringer solution to wash blood from the vessels. This was followed by a continuous addition of warm fixative (for a few seconds) which was then rapidly cooled (to 0° centigrade). When perfusion-fixation of the brain was desired, the dorsal aorta was clamped just after the introduction of the warm fixative. For the spinal cord, the whole neck only was clamped with a hemostat. The right atrium was opened to allow the release of the returning fluid.

Successful perfusion was indicated by blackened nervous tissue. From these animals some slices were taken to be embedded in paraffin for light microscopy. On the other hand, control and adjacent small areas of tissue were kept in the ice box in the same cold fixative to be embedded in open and glycol methacrylate for ultrastructural studies. The following special procedures were carried out:

a) For light microscopy the periodic acid Schiff (PAS) reaction was routinely employed. Pretreatment of control sections with dimedone was done according to Bulmer procedure,[4] as well as enzymatic digestion with alpha amylase before PAS. An adaptation of Scarcelli's technique for aldehydes with paraphenylenediamine[31] was also used.

b) For ultrastructural demonstration of glycogen a lead salt stain,[19] in some cases combined with uranyl acetate stain, was used.

c) For microscopic study of enzymatic digestion of glycogen osmium fixed material was embedded in plastic. Small pieces of glycogen body tissue of chicken spinal cord fixed by chromic osmium perfusion were embedded in glycol methacrylate.[22] Thick sections were cut with a Porter-Blum ultramicrotome, placed on glass slides, and covered either with saliva or with a 1-percent solution of alpha amylase during one hour at 37° C and then the PAS reaction was performed according to Munger procedure.[26] Adjacent sections were placed on glass slides and stained by the PAS method alone. Parallel sections were kept in water at 37° C for one hour and then the PAS reaction was performed.

RESULTS

Perfusion with buffered osmium tetroxide solutions provided excellent preservation of glycogen for light and electron microscopical studies. Nervous tissue fixed with osmium by perfusion, embedded in paraffin, and stained with PAS in a conventional way, exhibited a well-preserved and homogenously distributed glycogen. However, there was an extensive staining of the background due mainly to myelin that made the slide almost useless (Fig. 1A). This discouraging result could be avoided if a longer periodic acid oxidation and a pretreatment with dimedone were done. In this way only the glycogen stained positively by the PAS and an evaluation of its distribution could easily be done. This procedure has been very helpful in the study of chicken spinal cord (Fig. 1B) (to be compared with fixation by immersion with picroalcoholic fluid (Fig. 2)) as well as rat brain (Fig. 3). Osmium tetroxide perfusion proved to be very suitable for fixing glycogen not only in normal nervous tissue but in pathological material as well. Fig. 4 is a section of rat cerebral cortex from an animal exposed to 21.600 r of X-radiation to the head, sacrificed 24 hours later, and fixed by perfusion. Large amounts of glycogen are present in the ependymal cells and adjacent periependymal tissue. The absence of artifactual perivascular spaces is noteworthy. The glycogen is diffusely distributed in the perikarya of the periependymal astrocytes and in the perikarya and processes of the perivascular astrocytes.

Fig. 1A

Fig. 1B

Fig. 2

Fig. 3

Fig. 4

Likewise, large amounts of glycogen were present in the cerebellar cortex of the rat after X-ray irradiation. The glycogen appears mainly in the Purkinje cell layer. However, the Purkinje cells themselves are

spared (Fig. 5). Light and electron microscopy studies[8,12] proved that the glycogen was located mainly in the perikarya and processes of the von Bergmann cells. In irradiated nervous tissue of rat which was fixed by perfusion, embedded in epon, thin sectioned and stained with uranyl acetate and lead, the granules of glycogen were stained very deeply. In Figs. 6 and 7 perikarya and processes of astrocytes are shown, loaded with these granules. The glycogen is so abundant and its granules are so tightly packed that a better fixation cannot be expected.

Fig. 5

Figs. 6,7

Turning to the staining procedures after periodic acid oxidation, the glycogen was successfully stained with paraphenylenediamine as shown in Fig. 8 where only the glycogenic cells of the glycogen body of the chicken appear specifically stained.

Fig. 8

Difficulties arise when the diatase digestion of particulate glycogen is attempted for electron microscopy studies. The digestion of glycogen in tissue that has been fixed in osmium by perfusion and embedded in the plastic hydrosoluble media glycolmethacrylate is possible, without the removal of the plastic as is shown in Figs. 9 and 10. In sections treated in the same way with water there are no changes in the amount of glycogen in the sections. On the other hand, when the epon embedding media was used, all the tests of digestion were negative.

Figs. 9,10

DISCUSSION

Adequate fixation and identification of glycogen at the level of light microscopy and at the subcellular level are of increasing importance in studies of normal and altered nervous tissue. Proper fixation and preservation of the glycogen have raised numerous problems.

The advantages of picroalcoholic fluids are evident when chemical determinations are made of the glycogen preserved by different kinds of fixatives. But when we turn to the histochemical fixation of glycogen for combined light and electron microscopical studies, the achievement of good cellular fixation should be kept in mind. The utilization of aqueous fixatives then becomes imperative.

The over-all factors that determine the best fixation of glycogen are not yet completely established. For a long time it was thought that in order to preserve adequately the tissue glycogen, the fixative should precipitate it. This is the case when alcoholic fixatives are used. But this effect is accompanied by the production of artifacts due to the precipitation of the elementary glycogen particles in coarse aggregates. Other mechanisms seem also to be involved. It has been claimed that denatured protein matrix immobilizes and traps the glycogen. The picric acid present in many fixatives would act in this way.

The amount of glycogen preserved by aqueous fixation is not so great as that obtained after alcoholic fixation. However, according to Pearse[29] the solubility of glycogen in different fixatives has been too much exaggerated. Furthermore, Baker[3] states that formaldehyde fixes proteins in such a way that the glycogen is bound to them and cannot dissolve easily in water. For instance using Rossman's fluid, preservation of 99 percent of the glycogen content of the tissue has been reported.[9]

Vallance-Owen[35] showed that formaldehyde is as effective as absolute alcohol for glycogen fixation. He also showed that there is no loss of glycogen in tissue fixed 38 days and then washed in tap water for 12 hours. According to Pearse[29] the lack of solubility of fixed glycogen is probably

due to precipitation of a complex glycogen-protein. In fact, in its native state, glycogen is bonded to proteins by Van der Waals forces.

The mechanism of the preservation of glycogen by fixation with aqueous solutions of osmium tetroxide is not clear. It is certainly not dependent on a direct reaction between glycogen and osmium tetroxide. "In vitro" experiments by Bahr[2] have shown that such a reaction does not occur. Classically, osmium tetroxide was considered a poor fixative for carbohydrates; its slow penetration of the tissues being perhaps the main reason for this. However, electron microscopists have demonstrated the possibility of obtaining a reasonable preservation of glycogen after fixation of tiny pieces of tissue with osmic solutions.[5;11;20;30] Such procedure, however, has all the setbacks of fixation by immersion and is inadequate for combined light and electron microscopic studies. It is particularly unsuitable for experimental studies of the nervous system as it precludes the observation of relatively large areas, so necessary for accurate topographic location of cytological changes.

On the other hand, perfusion with osmium tetroxide solutions (particularly Dalton's fluid) allows a uniform and extensive fixation. This procedure eludes the main setback of osmium fixation, that is, the slow rate of penetration. Cells, fibers, vessels are quickly reached by the perfusate that simultaneously penetrates from multiple sites.

It is conceivable that as in the case of immersion of tiny tissue pieces, perfusion with osmic tetroxide will insure the preservation of glycogen by "trapping" it in the frame of the cell structures quickly fixed and stabilized by their reaction with osmium.

From our data it can be concluded that the fixation of the glycogen in normal and pathological nervous tissue, with aqueous fixatives by intravascular perfusion mainly with buffered osmium solutions, is adequate. The glycogen is not only preserved and distributed homogenously in the cells, but many artifacts are avoided, such as polarity and perivascular retraction. Furthermore, the tissue fixed in this way offers the possibility of a parallel study for light and electron microscopy of adjacent areas.

Enzymatic digestion of glycogen for electron microscopy was attempted in this osmium fixed material. It has been said that either osmium fixation or plastic embedding of tissue prevents the digestion of glycogen, as is the case for nucleic acids and some proteins. With the use of the glycol methacrylate as an embedding medium, the enzymatic digestion of glycogen has been successful without removal of the plastic from the sections. We may say that glycolmethacrylate embedding has paved the way for the enzymatic identification of glycogen in ultrathin sections which would be of extreme importance since it is very frequently necessary to differentiate the glycogen particles from ribonucleoprotein and viral particles. Preliminary tests in our hands have been successful and further data on this procedure will be reported elsewhere.[10]

The glycogen was successfully stained with paraphenylenediamine after periodic acid oxidation. If this procedure is followed by gold chloride toning, it would have some prospects for also becoming a technique for ultrastructural studies.

SUMMARY

The relative value of different methods for combined light and electron microscopical studies of glycogen in the nervous tissue was investigated.

Picroalcoholic fixatives preserve glycogen in a considerable amount but give an inadequate morphological image of glycogen distribution and are unsuitable for ultrastructural studies.

Fixation by perfusion with Dalton's chrome-osmic fluid seems adequate for ultrastructural cytochemistry of glycogen. Furthermore it permits routine paraffin embedding of brain slices adjacent to those used for electron microscopy.

Dimedone blocking is a necessary step for a selective staining of glycogen with PAS after osmic fixation.

Enzymatic removal of glycogen in osmic fixed nervous tissue can be done in paraffin embedded tissue. It can also be performed in glycol-methacrylate embedded tissue without removal of the embedding medium.

Paraphenylenediamine stains glycogen following periodic acid oxidation.

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LEGENDS

Fig. 1. Chicken spinal cord. Section at the upper pole of the glycogen body after fixation by perfusion with chrome-osmium solution and embedding in paraffin. A - PAS done in a conventional way. B - PAS after pretreatment with dimedone. Notice that after dimedone the glycogen (gly) close to the ependyma (ep) stains positively, being homogenously distributed. The adjacent nervous tissue (nt) remains unstained. X 90.

Fig. 2. Chicken spinal cord. Area similar to that in Fig. 1. Fixation by immersion in cold Rossman fluid, PAS staining, paraffin embedding. Note the coarse granular appearance of the glycogen (gly) close to the ependyma (ep). X 180.

Fig. 3. Rat brain fixed by perfusion with chrome-osmium solution 48 hours after 10.000 r X-radiation to the head. Paraffin embedding, PAS after dimedone. Notice the homogenous distribution of the glycogen (gly) in the ependymal cells (arrow) as well as in the periependymal astrocytes (ast). No artifacts occur around the vessels (v). X 608.

Fig. 4. Rat cerebral cortex fixed by perfusion with chrome-osmium solution 24 hours after 21.600 r X-radiation to the head. Paraffin embedding, PAS after dimedone. A - Glycogen is seen in the perikarya and processes of two astrocytes (ast) presumably of protoplasmic type. B - The absence of artifacts is evident as well as the dilation of the vessels (v) due to the perfusion procedure. X 760.

Fig. 5. Rat cerebellar cortex fixed by perfusion with chrome-osmium solution 24 hours after 21.600 r X-radiation to the head. Paraffin embedding, PAS stain. Area of Purkinje cell (pc) layer. The glycogen is shown in the cytoplasm of Bergmann cells (bc). Altered granule cells (gc) are evident. X 1550.

Fig. 6. Rat cerebellar cortex fixed by perfusion with chrome-osmium solution 24 hours after 21.600 r X-radiation to the head. Epon embedding. Uranyl acetate and lead staining. Area of Purkinje cells layer. The cytoplasm of a Bergmann cell close to the nucleus (bcn) is loaded with glycogen (gly). An unmyelinated nerve fiber (nf) is shown. X

Fig. 7. Rat cerebellar cortex fixed by perfusion with chrome-osmium solution 24 hours after 21.600 r X-radiation to the head. Epon embedding. Uranyl acetate and lead staining. Molecular layer of the cerebellar cortex. Swollen astrocytic process loaded with glycogen granules (gly) which stains deeply with lead. To the left a myelinated fiber (mf) and, to the right, an axo-dendrytic synaptic contact (syn) are seen. X

Fig. 8. Chicken spinal cord. Area of the glycogen body (gb). Fixation by immersion with Rossman fluid. Paraffin embedding. Paraphenylenediamine stain. Notice that the staining is specific for the glycogenic cells. Adjacent nervous tissue (nt) remain unstained. X 37.

Fig. 9. Chicken spinal cord. Section of glycogen body. Fixation by perfusion with chrome-osmium solution. Glycolmethacrylate embedding. Thick section stained with PAS without removal of the embedding medium. Notice the homogenous distribution of the intracellular PAS positive material. X 305.

Fig. 10. Thick section adjacent to that in Fig. 9. PAS stain after one hour of incubation at 37° C with a 1-percent alpha amylase solution. After the enzymatic digestion of the glycogen, the glycogenic cells do not stain with the PAS. X 305.

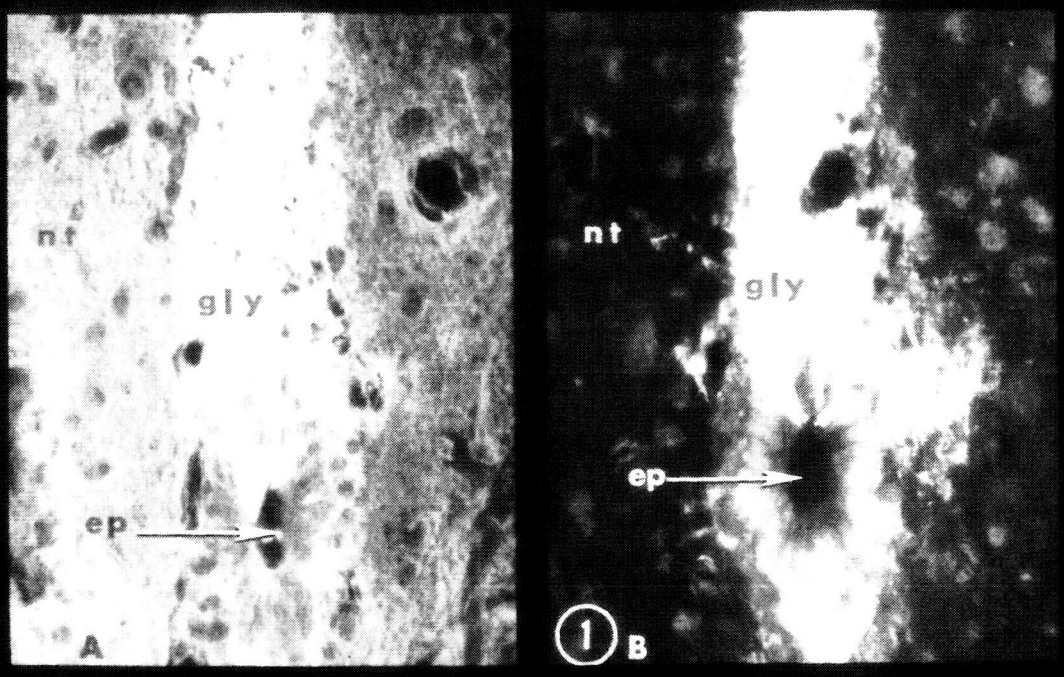
METHODS OF FIXATION

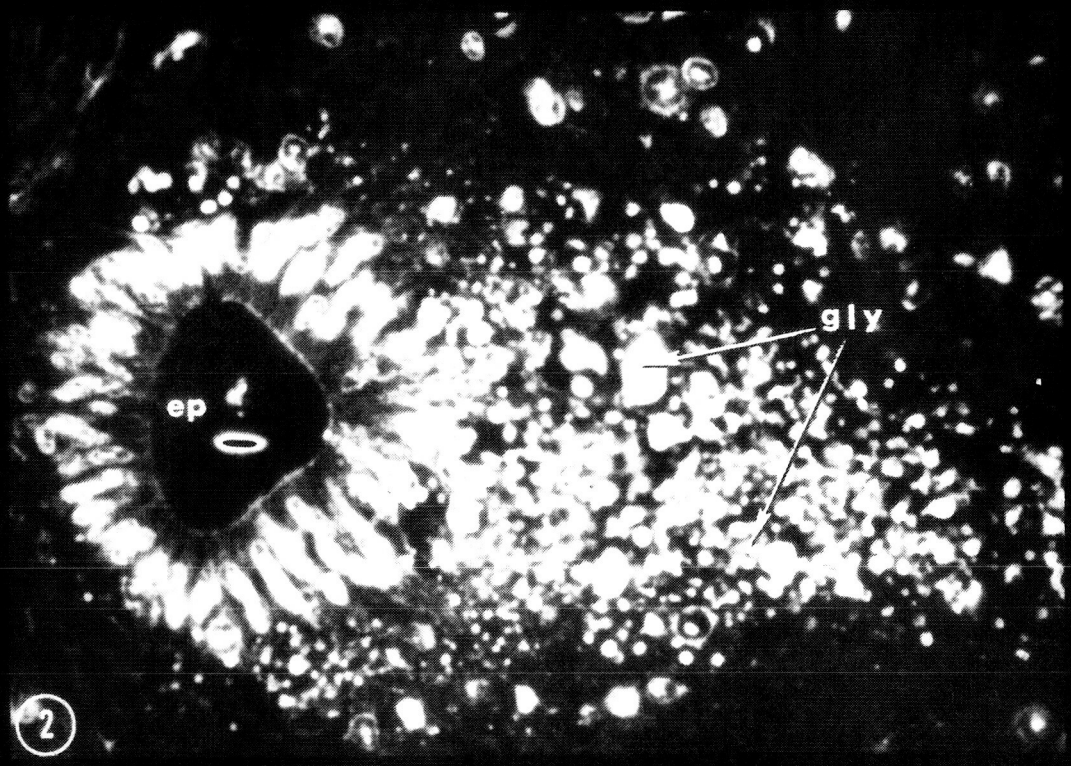
- I CRYOFIXATION — FREEZING DRYING
- II CRYOCHEMICAL FIXATION
  - FREEZING SUBSTITUTION
  - FREEZING THAWING
- III CHEMICAL FIXATION
  - a- BY IMMERSION
  - b- BY PERFUSION

Table I.- Different types of methods used for the fixation of glycogen

LIGHT MICROSCOPY ROUTINE	ELECTRON MICROSCOPY ROUTINE
FIXATION: IN ALCOHOLIC OR PICROALCOHOLIC SOLUTIONS (ROSSMAN, GENDRE, ETC)	FIXATION: IN AQUEOUS SOLUTIONS (OSMIUM TETROXIDE, PERMANGANATE)
EMBEDDING: IN PARAFFIN OR CELLOIDIN	EMBEDDING: IN PLASTICS
STAINING: BY PAS, BEST CARMINE AND IODINE IS POSITIVE FOR GLYCOGEN (THOUGH NO SPECIFIC)	STAINING: BY LEAD SALTS IS POSITIVE FOR GLYCOGEN (THOUGH NO SPECIFIC)
IDENTIFICATION: BASED ON ENZIMATICALLY DIGESTED CONTROL SECTIONS TAKEN FROM THE SAME BLOCK	IDENTIFICATION BASED ON: a. MORPHOLOGICAL FEATURES; b. ENZIMATIC DIGESTION OF DIFFERENT BLOCKS OF TISSUE; c. LIGHT MICROSCOPY OF ADJACENT THICK SECTIONS

Table II.- Routines usually followed for light and electron microscopy studies of glycogen





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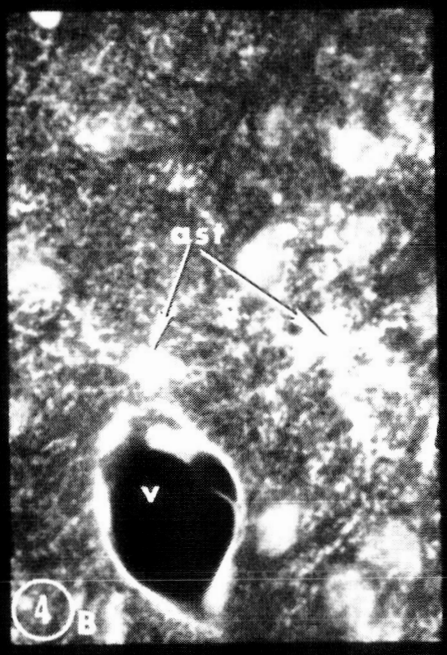

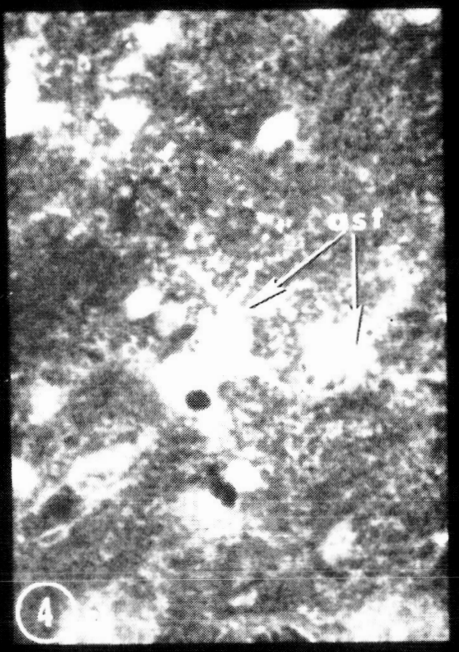

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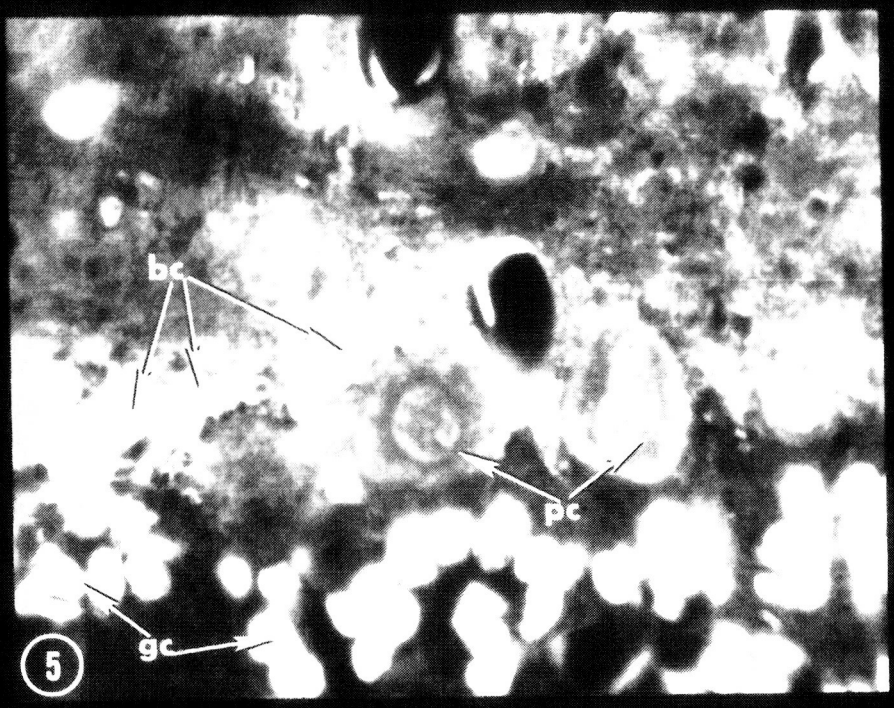

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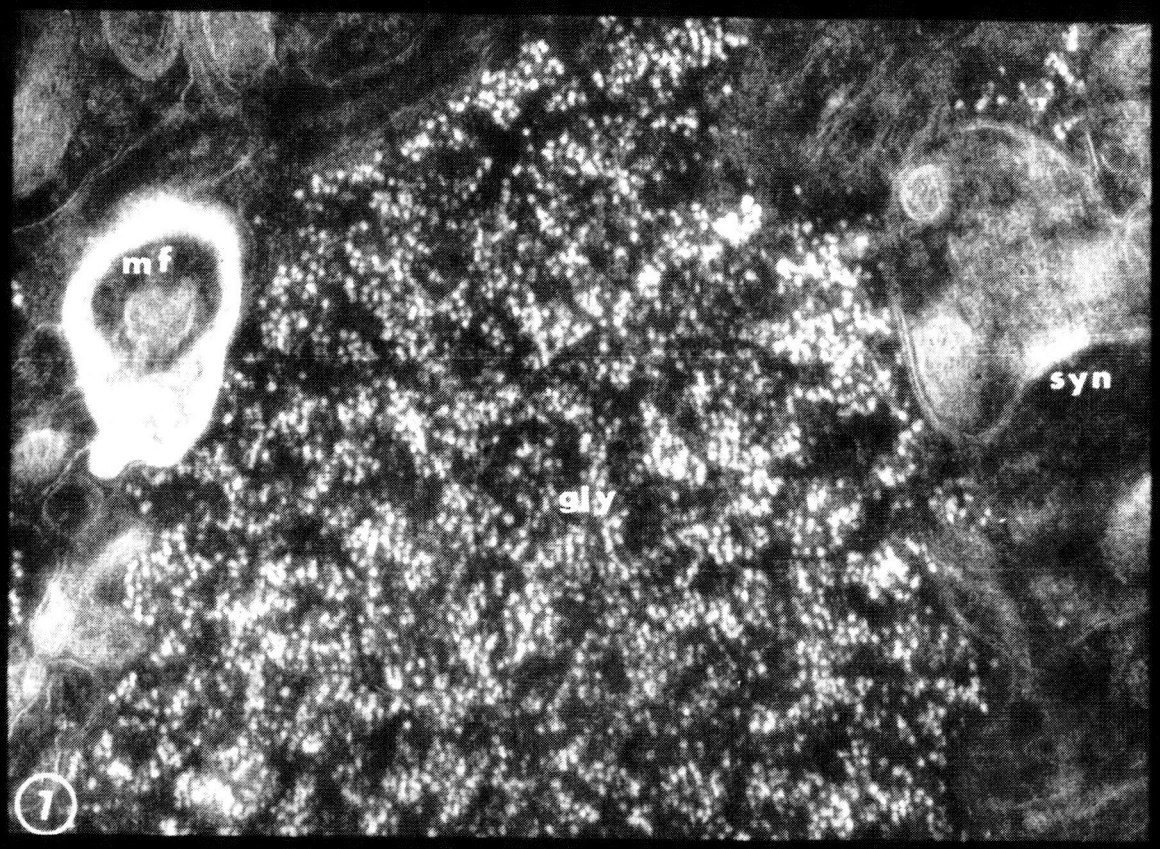

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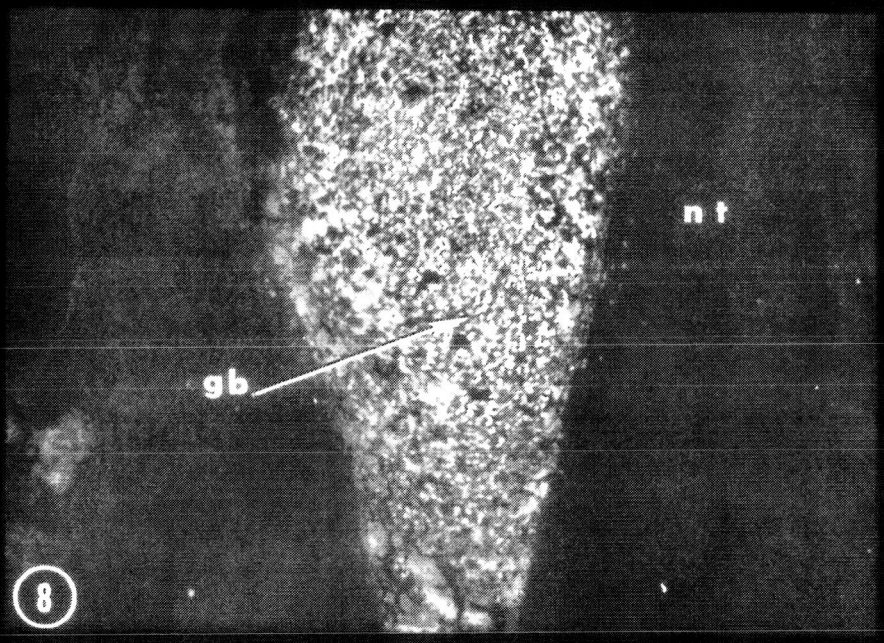

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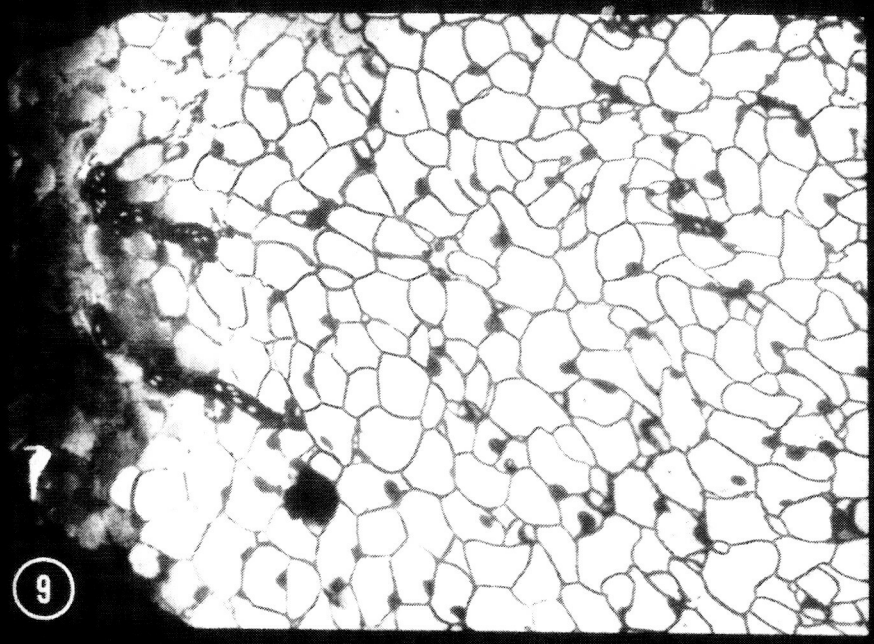

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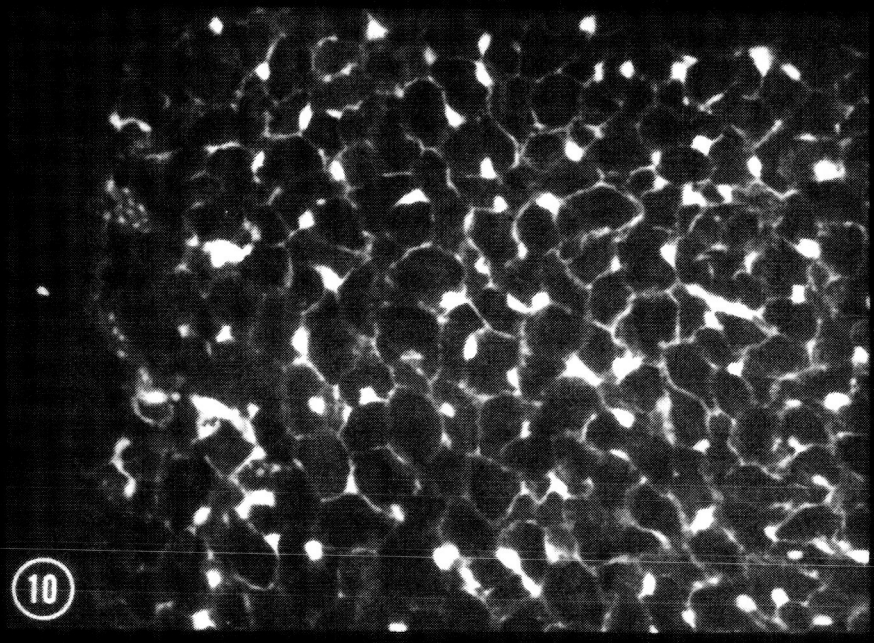

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